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Assessing the toxicity of triphenyltin to different life stages of the marine medaka *Oryzias melastigma* through a series of life-cycle based experiments

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ABSTRACT

Toxic effects of triphenyltin (TPT) to different life stages of the marine medaka *Oryzias melastigma* were investigated through a series of life-cycle based exposure experiments. In embryo stage, TPT exposure could elevate the heartbeat rate at Day 6–8 post-fertilization and increase the expression levels of five heart development related genes (i.e., *ATPase*, *COX2*, *BMP4*, *GATA4* and *NKX2.5*). In larval stage, TPT shortened the body length at ≥ 10 μg/L and suppressed the swimming activity of the fish larvae at Day 1 post-hatching at 50 μg/L. In reproductive stage, TPT exposure resulted in a male-biased sex ratio (2 μg/L) and reduced the gonadosomatic index (GSI) in females (≥ 0.1 μg/L), which might in turn lead to a decline in their population fitness. The reproductive stage of *O. melastigma* was more sensitive to TPT than other stages, while the GSI of female medaka was the most sensitive endpoint.

1. Introduction

Small freshwater fishes such as the zebra fish (*Danio rerio*), the fathead minnow (*Pimephales promelas*) and the Japanese medaka (*Oryzias latipes*) have been commonly applied as model organisms for ecotoxicological researches for more than two decades (Dodd et al., 2000; Fent and Meiyer, 1994; Wittbrodt et al., 2002). On the contrary, a saltwater fish model was virtually unavailable over the past decades. Only recently, the marine medaka *Oryzias melastigma* has been suggested and applied as a small saltwater fish model for assessing chemical toxicity (e.g., nano zinc oxide, perfluorooctane sulfonate) and environmental stresses (e.g., hypoxia) in the marine environment (Huang et al., 2011; Kong et al., 2008; Wong et al., 2010; Kim et al., 2016). The marine medaka, originated from India and hence also called Indian medaka (Menon, 1999), can adapt to a wide range of salinity and grow well in different seawater conditions. It has a number of attributes which are essential for a model species, including (1) small size; (2) short generation time; (3) sexual dimorphs; (4) easy to culture and breed; and (5) phylogenetically close to its freshwater counterpart, the Japanese medaka *O. latipes*, of which the entire genome information has been available (Kasahara et al., 2007; Kong et al., 2008). In recent years, a number of documented studies have adopted *O. melastigma* as a marine fish model to investigate the toxicity and molecular toxic mechanisms of various anthropogenic pollutants such as water-accommodated fractions (Rhee et al., 2013), pentabromodiphenyl ether 47 (van de Merwe et al., 2011), inorganic mercury (He et al., 2012; Wang et al., 2011), benzotriazole (Bao et al., 2011), antifouling biocides (Weis and Weis, 1989) and nano zinc oxide (Wong et al., 2010).

Studies on the toxicity of environmental pollutants to fish species have been mainly conducted for their partial life-cycles such as embryonic stage, early fish larval stage and reproductive stage (Strmac and Braunbeck, 1997). However, most of these partial life-cycle toxicity tests have their own limitations. Firstly, toxicity tests using fish embryos mainly focused on the development (e.g., eye, heart or body development) and the hatching rate of embryos, but it might ignore possible consequences in later stages of the fish that caused by the abnormal development in the embryonic stage (i.e., latent toxic effect). For example, exposure of fish embryos to crude oil in embryonic stage could alter cardiac morphology which in turn led to a reduction of aerobic capacity in adult fish (Hicken et al., 2011). Secondly, the early fish larval stage is generally very sensitive to environmental pollutants and thus has been widely applied in toxicological studies (Weis and Weis, 1989). Nonetheless, short-time exposure of fish larvae to environmental pollutants may result in different conclusions when compared with the results generated from chronic exposure studies. It has been argued that the exposure period has a major implication on the growth inhibition test, which is because growth might be temporarily stalled during early stages of exposure and a long-term acclimation to chemicals would possibly lead to compensation for any deleterious effects (Wong et al., 2010). Thirdly, not only the reproduction capacity of the

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fish, but also the sex ratio can dramatically affect the population dynamics. A full life-cycle exposure of fish to environmental pollutants may cause male- or female-biased sex ratios and suppress the gonadal development, which can in turn affect the fish at population level (Devlin and Nagahama, 2002). These effects, however, may not be reflected by a toxicity test focusing on the reproductive stage only. Therefore, the full life-cycle toxicity test is more valuable and definitive for determining adverse effects of environmental pollutants, given that the fish is exposed to chemical contaminants in ecosystems at different life stages throughout their life span. Nonetheless, chronic toxicity tests or full life-cycle toxicity tests, especially for fish species, have been rarely conducted because of huge workload and time-consuming processes (Srivastava and Olson, 2000). To date, there has been no information on the toxicity of organotin compounds (OTs) on the full life-cycle of marine fish.

The cardiovascular system is often the first to become functional in developing vertebrate embryos. The development of fish heart is susceptible to environmental stresses because the formation of the heart involves a precisely orchestrated series of molecular and morphogenetic events. Thus, even subtle perturbation of this process can have catastrophic consequences in the heart development (Srivastava and Olson, 2000). The heart development is a dynamic process that is regulated by many genes (Olson, 2006). Several genes that involved in the heart development in the marine medaka *O. melastigma* has been cloned recently, and these genes are GATA-binding protein4 (*GATA4*), NK2 transcription factor related 5 (*NKX2.5*), bone morphogenetic protein (BMP4), fibroblast growth factor 8 (FGF8), Na^+-K^+-ATPase (*ATPase*), erythropoietin (*EPO*), cyclooxygenase-1 (*COX1*) and cyclooxygenase-2 (*COX2*) (Huang et al., 2012). This genomic information enables us to investigate the effect of environmental stressors on the heart development of *O. melastigma* embryos at molecular level.

Phenyltin (PT) compounds, in particular triphenyltin (TPT), have been commonly applied as effectual biocides for various industrial and agricultural purposes such as antifouling biocides on ship hulls, and fungicides on wood products (Yi et al., 2012). Due to their intensive and wide usages, occurrences of PTs have been reported in different compartments of the marine environment such as coastal seawater, sediments and biotas (Hu et al., 2006; Rantakokko et al., 2010; Wang et al., 2008). Recently, an increasing trend of TPT contamination has been reported in Asian coasts such as the marine environment of Hong Kong (Ho and Leung, 2014), Taiwan (Meng et al., 2005) and Xiamen, China (Xie et al., 2010).

Toxicological studies of TPT have been well summarized in a review article (Yi et al., 2012). As for fish species, the median lethal concentration (LC_{50}) of TPT ranged from 7.1 (the fathead minnow *Pimephales promelas*) to 62 μg/L (the goldfish *Carassius auratus*) (Jarvinen et al., 1988; Johnson and Finley, 1980). In addition, a chronic growth inhibition test was carried out using larvae of the fathead minnow *Pimephales promelas*, and the 30-day EC_{50} of TPT was reported at 0.23 μg/L (Jarvinen et al., 1988). However, most of the available toxicological information was biased to freshwater fish species and the knowledge about the toxicity of TPT to saltwater species is still lacking.

This study, therefore, aimed to investigate the toxic effect of TPT on the marine medaka through a combination of physiological and life-cycle approaches. In addition, gene expression analyses were conducted for embryos at Day 6 post-hatching with a special focus on the regulation of the genes related to the heart development.

2. Materials and methods

2.1. Test chemical

A stock solution of TPT chloride (TPTCl) was prepared by dissolving TPTCl (>95%; Sigma, USA) in dimethyl sulfoxide (DMSO; ACS reagent, 99.9%; Sigma, USA). Test solutions (at concentrations ranging from 0.1 to 50 μg/L) were made by spiking appropriate volumes of the TPTCl stock solution into filtered artificial seawater (FASW; salinity: 32 ± 1 ppt; filter membrane: 0.8 μm, Millipore, USA).

2.2. Life-cycle exposure of the fish to TPTCl

The marine medaka *O. melastigma* were obtained from the medaka culture facility at the School of Biological Sciences, The University of Hong Kong, Hong Kong, China. Eggs spawned from female marine medaka were carefully collected within 24 h after fertilization and then subjected to TPTCl exposure. The exposure conditions throughout the whole experiment were kept constant at temperature: 25 ± 1 °C; salinity: 32 ± 1 ppt; and light-dark cycle: 14 h-light:10 h-dark.

2.2.1. Embryo stage

The exposure to TPTCl was started with embryos <24 h post-fertilization. Thirty embryos were exposed to 100 mL test solutions with different concentrations of TPTCl. Both seawater control (filtered artificial seawater, FASW) and solvent control (0.05% dimethyl sulfoxide (DMSO) in FASW) groups were run in parallel. There were three replicates for each treatment or control group, and the test solutions were renewed every 48 h. The development of embryos was monitored every 24 h under a stereomicroscope (WILD MZ8, Leica, German), and any dead embryos were discarded. In order to investigate the effect of TPT on cardiac development of the fish, at Day 6 to Day 8 post-fertilization, five individuals were randomly selected from each replicate and their heartbeats were measured under a stereomicroscope (WILD MZ8, Leica, German). Both the number of hatched embryos and the time to hatch were monitored daily. After hatching, fish larvae were transferred into 2 L test solutions in glass tanks under the same condition for further exposure.

A separate exposure experiment was conducted under the same condition as mentioned above to collect samples for studying the expression of genes related to the heart development at Day 6 post-fertilization. In brief, 20 embryos were sampled from each replicate of each treatment or control, and stored with 1 mL RNA*later* solution (Life Technologies, USA). The collected embryos were then homogenized with a tissue pestle and RNA samples were extracted with RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's guidance. RNA concentrations were measured with Nanodrop 2000 (Thermo Scientific, USA) and the integrity of RNA samples was verified by 1.5% agarose gel. One microgram of each isolated RNA sample was applied in a total reaction volume of 20 μL using High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA) for complimentary DNA (cDNA) synthesis. For real-time quantitative PCR, 2 μL diluted (×5 times) reverse-transcripted cDNA samples and 250 nM primer sets were applied in each reaction, which was performed on a CFX96 Real-Time System (Biorad, CA, USA) with SYBR Green (iQ™ SYBR Green Supermix, Biorad, USA) as fluorescence dye. Primer sequences of the selected genes are listed in Table 1.

2.2.2. Larval stage

Photos of the randomly selected larvae (5 larvae in each replicate of each treatment) were taken at Day 1 post-hatch using a dissecting microscope equipped with a camera, and total length (TL) of each fish larva was measured from the images using the ImageJ (version 1.44, National Institute of Health, USA).

The swimming activities of the larvae at Day 1 post-hatch were monitored according to the method described in elsewhere with slight modifications (Teather et al., 2005). In brief, 5 fish larvae were randomly selected from each replicate and placed individually in clean 50 mL petri dishes with 20 mL of test solutions. Each fish larva was acclimated in the petri dish for 2 min, after which it was videotaped with a camera mounted directly above the petri dish for 2 min. The path travelled by each fish within 2 min was traced on a 14-inch monitor, and the track were then scanned and converted to linear distance (unit: cm) using ImageJ software (version 1.44, National Institute of Health, USA).

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